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Quantification of the biocontrol agent *Pseudomonas fluorescens* Pf153 in soil using a quantitative competitive PCR assay unaffected by variability in cell lysis- and DNA-extraction efficiency

Davide Gobbin[a,b,*], Fabio Rezzonico[a,c], Cesare Gessler[a,b]

[a]*SafeCrop Centre, Istituto Agrario di S. Michele all'Adige, via Mach 1, 38010 S. Michele all'Adige, TN, Italy*
[b]*Plant Pathology, Institute of Integrative Biology, Swiss Federal Institute of Technology, Universitätstrasse 2/LFW C16, 8092 Zürich, Switzerland*
[c]*Agroscope ACW Changins-Wädenswil, Schloss, 8820 Wädenswil, Switzerland*

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Abstract

Although often neglected, variability in cell lysis efficiency and DNA extraction yield represents the major hurdles of any polymerase chain reaction (PCR)-based quantification protocol in soil and other natural environments. In this study we developed a technique that minimizes the effects of these constraints, providing at the same time a reliable internal control to distinguish between PCR-inhibition and negative results. We used *Pseudomonas fluorescens* Pf153, a root-colonizing bacterium that shows biocontrol activity against tobacco and cucumber black root rot, as the target organism for PCR quantification. Prior to DNA extraction, the genetically engineered, cognate reference strain *P. fluorescens* CHA0/c2 was inoculated in a reference soil. CHA0/c2 in the reference soil and Pf153 in the soil sample were lysed in parallel and afterward the lysates were mixed in known proportions. CHA0/c2 carries the plasmid pME6031-cmp2 that contains an allelic variant (competitor) of the Pf153 specific sequence Pf153_2. In a quantitative competitive PCR (QC-PCR) assay the competitor allows the quantification of the target strain down to 0.66 Pf153 CFU/mg soil. Processing the reference strain in the same way as Pf153 enables the exact quantification of the target strain in biocontrol assays performed in natural soil, overcoming differences in DNA extraction efficiency and PCR amplification from different soil environments. This technique is easily adaptable to other *Pseudomonas* strains simply by replacing the competitor used here with one derived from a SCAR-marker which is specific for the strain of choice.
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Keywords: Biocontrol; DNA extraction internal control; Monitoring; Quantitative competitive PCR; *Pseudomonas fluorescens*; Risk assessment

1. Introduction

Biological control agents are an alternative to the use of fungicides, for suppression of fungal pathogens in agricultural production (Sigler et al., 2001). Among the many organisms suited for biocontrol of soil borne diseases, the root-colonizing bacterium *Pseudomonas fluorescens* CHA0 has become a model for studying the behavior of biocontrol inoculants in the soil ecosystem (Rezzonico et al., 2003). A closely related strain, *P. fluorescens* Pf153 shows similar biocontrol attributes (Fuchs and Défago, 1991). It was isolated from the roots of tobacco grown in Morens soil (Fribourg canton, Switzerland), which is suppressive to black root rot of tobacco mediated by *Thielaviopsis basicola*. Strain Pf153 also inhibits *Phomopsis sclerotioides* L3 on PDA and King's B agar (Fuchs et al., 2000), *Botrytis cinerea* and *Phytophtora infestans* (D. Stephan, personal communication). Antifungal compounds synthesized by Pf153 include an extracellular protease and hydrogen cyanide. Unlike other pseudomonads from Morens soil (e.g. strain CHA0), Pf153 does not produce 2,4-diacetylphloroglucinol or pyoluteorin (Fuchs and Défago, 1991).

*Corresponding author. Plant Pathology, Institute of Integrative Biology, Swiss Federal Institute of Technology, Universitätstrasse 2/LFW C16, 8092 Zürich, Switzerland. Tel.: +4144 632 31 17; fax: +4144 632 15 72.
E-mail address: davide.gobbin@agrl.ethz.ch (D. Gobbin).

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In order to properly understand the biocontrol performance and persistence of *P. fluorescens* Pf153 two main requisites are needed: unequivocal strain identification and a rapid means for monitoring its population dynamics. Highly sensitive diagnostic assays, based on the polymerase chain reaction (PCR) of target-specific sequences, have been implemented successfully for the identification of important biocontrol agents (Hermosa et al., 2001; Pujol et al., 2005). Despite wide research into genetic diversity of *Pseudomonas* genes, such as polyketide synthase genes (*phlD*) (Wang et al., 2001), hydrogen cyanide (*hcnBC*) synthesis genes (Ramette et al., 2006) or response regulator gene *gacA* (De Souza et al., 2003), no information is available about specific sequence-characterized amplified regions (SCAR markers) for *P. fluorescens* Pf153.

For monitoring the population dynamics of Pf153, a method for quantifying its population density in soil is essential. Before the widespread use of the real-time PCR technique, the usual approach to quantify target sequences within the PCR framework was the quantitative competitive PCR (QC-PCR) assay, which has been used for quantifying viruses (Piatak et al., 1993), bacteria (Li and Drake, 2001), to identify genetically modified organisms (Studer et al., 1998) and for quantifying gene expression (Chung et al., 2002). This technique employs a competitive DNA as an internal standard; the competitive template is an allelic variant of the target template that provides a stringent internal control in the amplification process. Quantification is based on determining the amount of the amplified products derived from the target and competitive templates, as replicated proportions of the target template are co-amplified with the dilution series of the internal standard template. Relative amounts of target to competitor are preserved during amplification and can be compared using quantitation analysis softwares (Schnell and Mendoza, 1997). QC-PCR is more labor-intensive than real-time PCR but requires only ordinary laboratory equipment and it is more cost-effective (Collantes-Fernández et al., 2002).

However, the reliability of both methods finally depends on a qualitatively and quantitatively reproducible DNA extraction technique, a factor which is often neglected in many works published in this field (Mumy and Findlay, 2004). Soils are very heterogeneous environments characterized by different ionic potentials and variable amounts of salts, organic matter, clay, silt, sand, etc. For this reason the same DNA extraction method may display remarkably different yields (Mumy and Findlay, 2004), as polyphenols and humic acids present in the sample strongly interfere with this procedure. Efforts to quantify microorganisms in soil without knowing the yield of DNA extraction are very questionable and the subsequent comparison of soil colonization in different environments is hardly achievable (Miller et al., 1999).

Therefore, in order to perform risk assessment studies of Pf153, following three objectives were fixed: (1) the design of at least one reliable genetic marker allowing discrimination of Pf153 among soil colonizing microorganisms by SCAR technology, (2) based on one SCAR marker, the development of a QC-PCR for the robust and reliable quantification of Pf153 in soil and (3) the establishment of a soil DNA extraction methodology able to overcome differences in DNA extraction yields from different soils. These goals were achieved thanks to a genetically engineered reference *Pseudomonas* strain containing the competitor for QC-PCR added to each sample in the first phase of soil DNA extraction.

2. Materials and methods

2.1. Bacterial strains and RAPD amplification

The bacterial strains used in this work originate from different geographic regions worldwide (Table 1) and were available at the department of Plant Pathology, Institute for Integrative Biology at the ETH Zürich, Switzerland. Each strain was cultured in 10 ml liquid LB medium at 27 °C for 24 h. Cell suspensions (5 µl) were lysed at 95 °C for 5 min with 45 µl of sterile water. The quality of the DNA contained in the cell lysate was tested by PCR using the primers PSM_G and 9-27 (Table 2) targeting the 16S rRNA as described by Johnsen et al. (1999). An amplification product indicated that the DNA was sufficiently clean to allow further PCR and random amplified polymorphic DNA (RAPD) assays.

A RAPD–PCR amplification of cell lysate was carried out. Twelve arbitrary decamers (A2, B18, D7, E9, F6, F15, F20, H15, K3, J7, X8, Z15; Operon Technologies) were used to recover genetic diversities among the bacterial strains. RAPD–PCR amplification was carried out in 11 µl reaction mixtures containing: 1 µl of cell lysate plus 10 µl reaction mix (0.1 mM dNTP, 1.5 mM $MgCl_2$, 0.3 µM of each RAPD primer, 0.7 U Taq polymerase in 1 × PCR buffer) (Pharmacia Biotech, Uppsala, Sweden). Amplification conditions were as follows: two cycles, each consisting of 30 s at 94 °C, 30 s at 36 °C and 2 min at 72 °C; 20 cycles, each consisting of 20 s at 94 °C, 15 s at 36 °C, 15 s at 45 °C and 2 min at 72 °C; 18 cycles, each consisting of 20 s at 94 °C (after each cycle 1 s more), 15 s at 36 °C, 15 s at 45 °C and 2 min at 72 °C (after each cycle 3 s more). After the 40th cycle, a final extension step of 10 min at 72 °C was performed.

2.2. Conversion of RAPD fragments into SCAR markers

Amplified fragments were separated on a 1.5% agarose gel. Pf153-specific bands were excised and purified by means of the Wizard SV Gel purification system (Promega, Madison, WI) following the manufacturer's instructions. The purified DNA fragments were ligated to the pCR 2.1 TOPO vector and transformed into One Shot TOP10 cells (Invitrogen, Carlsbad, CA) using the TOPO XL PCR Cloning Kit System (Invitrogen, Carlsbad, CA) according to the manufacturer's recommendations.

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